Supplemental Figure 1A: Bortezomib induces the accumulation of TE1, TE3 and TE10 ESCC cells in the sub-G1 phase of the cell cycle. Cells were treated with bortezomib (10 nM) for 48 hrs, after which time cells were fixed and stained with propidium iodide. Cells were then analyzed by flow cytometry,

Supplemental Figure 1B: The proteasome inhibitors MG-132 and PI1 reduce the growth and survival of TE1 ESCC cells in 3D spheroid culture. Cells were treated with either MG-132 or PI1 (0, 0.3, 1 or 3 uM) for 72 hours. After this time, spheroids were stained with LIVE/DEAD assay kit and were photographed using an inverted microscope. Red = dead cells and green = live cells. Magnification X10.

Supplemental Figure 1C: The proteasome inhibitor MG-132 (300 nM) induces cleavage of caspase-3, -9 and PARP in the TE1 ESCC cell line. Cells were treated with MG-132 for increasing periods of time (0, 2, 6, 24 hrs), after which protein was extracted, resolved and subject to Western blotting. Actin confirms equal protein loading.

Supplemental figure 2: Treatment of ESCC cells with Bortezomib does not induce the formation of reactive oxygen species (ROS).

A: TE12 cells were treated with 500 μM H_2O_2 (as a positive control, 1hr), 10 nM Bortezomib (24 hrs), or normal saline (24 hrs) before being trypsinized and incubated with 100 nM CM-H_2DCFDA. Cells were then washed and resuspended in phosphate-buffered saline (PBS). Cellular ROS was measured by flow cytometry. No ROS

production was observed in cells treated with Bortezomib in comparison to the normal saline control. Data shown is representative of three independent experiments.

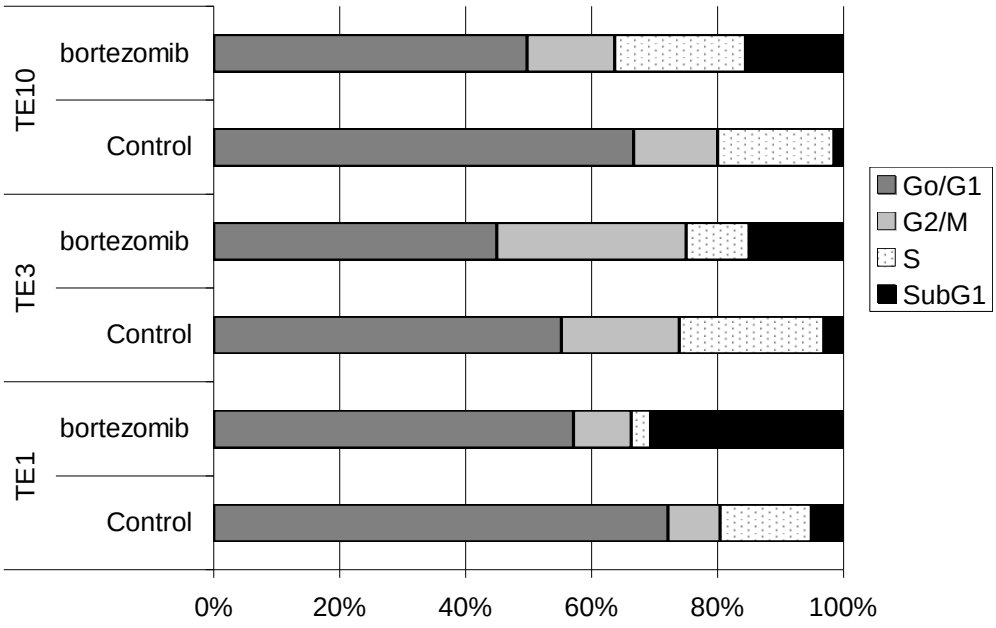
Supplemental Figure 3: JNK activity does not regulate Bortezomib-induced apoptosis.

Western blot showing upregulation of phospho-JNK following Bortezomib treatment (10nM) (upper panel). Cells were treated with JNK inhibitor VIII (1 μM), Bortezomib (10 nM), or a combination of the two drugs (lower panel). Pre-treatment of the cells with the JNK inhibtor did not affect the Bortezomib-induced cleavage of caspase-3 or induction of Noxa expression. Equal protein loading was confirmed by the β-actin loading control.

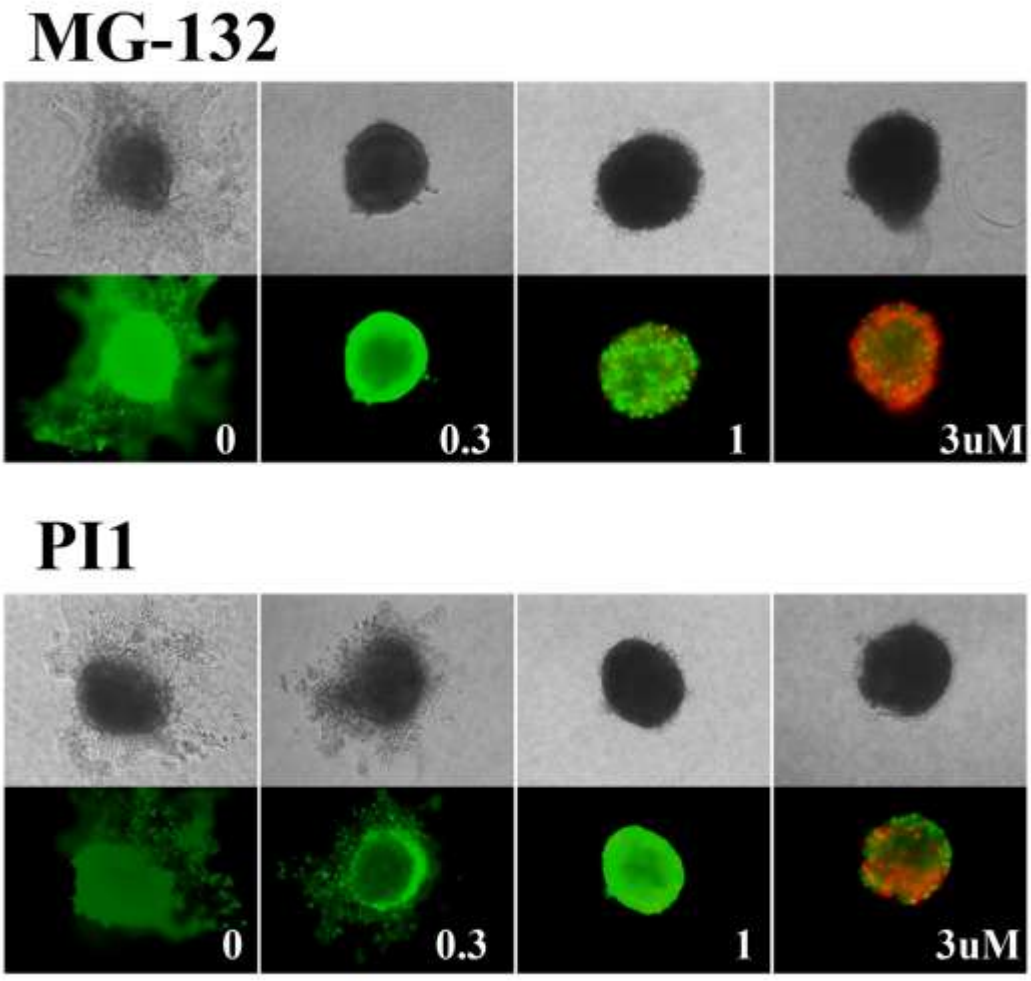
Supplemental Figure 4: Suggested scheme showing the mechanism of Bortezomib-induced apoptosis in human ESCC.

Supplemental Table 1: Only proteasome inhibitors are able to inhibit ESCC growth and survival in 2D and 3D cell culture models.

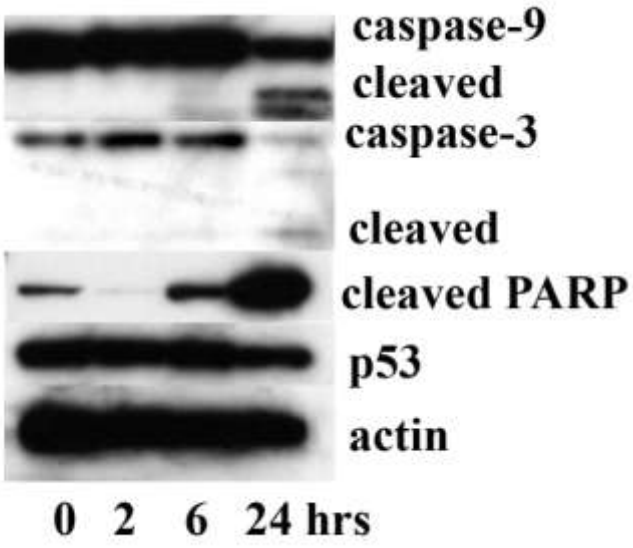
TE1, TE8, TE10, TE11, and TE12 cell lines were treated with various inhibitors (1 nM – 10 μM, 72 hrs) in two-dimensional (2D) and three-dimensional cultures (3D) as described in the materials and methods. * represents IC_{50}<3μM. Apoptosis was observed in cell lines using various inhibitors in a 2D context, but were found to be resistant when cultured in a 3D tissue-like structure.



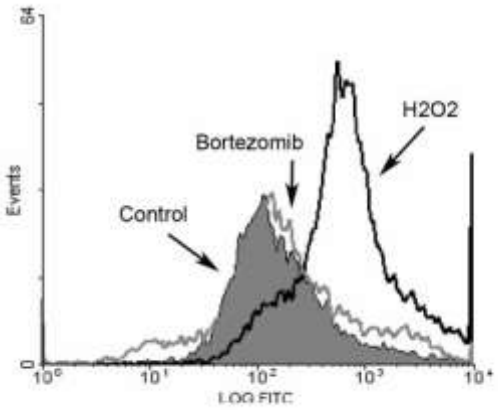
Supplemental Figure 1A:



Supplemental Figure 1B:



Supplemental Figure 1C:



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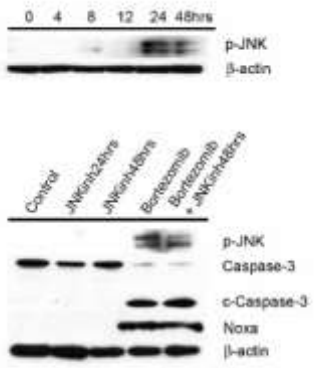
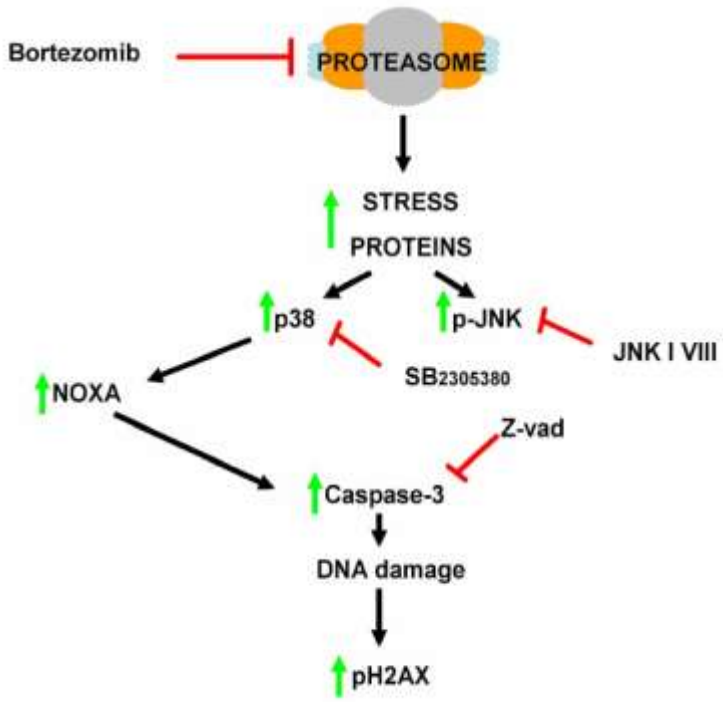


Supplemental Figure 3:



Supplemental Figure 4:

Table 1

Drug	Class	2D*	3D*
U0126	MEK	-	-
DW1/2	GSK3β	+	-
LY294002	PI3K	-	-
PI1	Proteasome inhibitor	+	+
MG-132	Proteasome inhibitor	+	+
Cyclopamine	Hedgehog	+	-
Bortezomib	Proteasome inhibitor	+	+
NS-398	Cox-2 inhibitor	-	-